

*Application
for
United States Letters Patent*

To all whom it may concern:

Be it known that **Cy A. Stein, Luba Benimetskaya and Nancy Guzzo-Pernell**

have invented certain new and useful improvements in

**PEPTIDES THAT DELIVER ANTISENSE OLIGONUCLEOTIDES WHICH DOWNREGULATE
PROTEIN EXPRESSION IN CELLS**

of which the following is a full, clear and exact description.

PEPTIDES THAT DELIVER ANTISENSE OLIGONUCLEOTIDES WHICH
DOWNREGULATE PROTEIN EXPRESSION IN CELLS

5

Background Of The Invention

10 Throughout this application, various publications are
referenced in parentheses by arabic numbers. Full citations
for these references may be found at the end of the
specification immediately preceding the claims. The
disclosures of these publications in their entirety are
hereby incorporated by reference into this application to
more fully describe the state of the art to which this
invention pertains.

15 Cancer is the second leading cause of death in the United
States. When cancer has metastasized, it can only be cured
by systemic therapy, usually cytotoxic chemotherapy.
20 However, it is often true that in this case chemotherapy is
ineffective. There are currently attempts to develop new
anti-cancer agents, including antisense oligonucleotides.
Antisense oligonucleotides can specifically inhibit the
translation of mRNA into protein. However, their use as
25 therapeutic agents is limited because of intracellular
transport and compartmentalization problems.

30 Thus, there are difficulties associated with delivery of
antisense oligonucleotides to their targets. At least in
tissue culture, antisense oligonucleotides almost invariably
must be condensed with a delivery reagent to ensure adequate
cellular uptake and release from sequestered sites in the
endosomes/lysosomes. The most commonly employed delivery

reagents are cationic lipids (e.g., Lipofectin), but these reagents may contribute their own cytotoxic effects, which affects the phenotype produced after treatment of the cells with the antisense effector molecule.

5

Accordingly, a large number of peptide delivery vehicles for antisense oligonucleotides have been devised. There are at least 20 peptides that increase the delivery of oligonucleotides to cells. These include pH sensitive fusogenic peptides, Antennapedia-type peptides, and the HIV tat C-terminus peptide. Other peptides that have been covalently conjugated to oligonucleotides include the ER-retaining peptide YKDEL (1) (SEQ ID NO:8), and various nuclear localization signal peptides (including the PKKKRKV sequence, SEQ ID NO:9, derived from the SV40 large-T antigen (2,3,4,5).

10

15

Non-covalent peptide oligonucleotide complexes have also been employed to increase cellular delivery (reviewed in 6). Morris, et al. (7) used a 27-mer peptide, called MPG, which was composed of the N-terminal domain of the HIV gp41 fusion sequence fused to the C-terminal domain derived from the nuclear localization signal derived from the SV40 large-T antigen has. Nuclear localization of oligonucleotide in fibroblasts was observed. Pichon, et al. (11) employed a permeabilizing peptide derived ultimately from an analog of the N-terminal sequence of the HA2 subunit of the influenza virus hemeagglutinin. Permeabilization was successful as judged by the nuclear localization of a fluoresceinated oligonucleotide. Pichon et al. (12) subsequently employed histidylated oligolysines to deliver antisense oligonucleotides targeted to ICAM-1, and demonstrated excellent antisense activity. However, in almost all the

25

30

Summary Of The Invention

This invention provides a peptide comprising consecutive amino acids, the sequence of which amino acids is shown in
5 SEQ ID NO: 2 and a composition comprising a complex between the peptide and an oligonucleotide, for example an oligonucleotide which comprises consecutive nucleotides having the sequence shown in SEQ ID NO:5 or SEQ ID NO:6, an
10 oligonucleotide which comprises a sequence capable of inhibiting translation of a mRNA into a protein, an oligonucleotide which comprises phosphorothioate linkages, an oligonucleotide which comprises between 10 and 40 consecutive nucleotides and an oligonucleotide that
15 comprises more than 40 consecutive nucleotides. This invention provides the instant peptide, wherein the peptide is membrane permeable.

This invention also provides a method of delivering an oligonucleotide into a cell comprising:

- 20 a) first contacting the cell with a lysosomotropic agent, and
b) then contacting the cell with the composition, under conditions permitting the composition to enter the cell and thereby deliver the oligonucleotide into the cell.
25 The invention provides the instant method wherein the lysosomotropic agent is chloroquine.

This invention also provides a method of inhibiting expression of a protein in a cell comprising delivering an
30 oligonucleotide into the cell using the instant method, under conditions permitting the oligonucleotide, once inside the cell, to hybridize with a nucleic acid encoding the protein and thereby inhibit expression of the protein from

the nucleic acid in the cell, for example inhibiting expression of Protein Kinase C alpha in for example a mammalian cell, which may be of human origin and/or cancerous. The nucleic acid may be a deoxyribonucleic acid or a ribonucleic acid such as a messenger ribonucleic acid.

This invention provides a peptide comprising consecutive amino acids, the sequence of which amino acids is shown in SEQ ID NO: 1 and a composition comprising a complex between the peptide and an oligonucleotide, for example an oligonucleotide which comprises consecutive nucleotides having the sequence shown in SEQ ID NO:5 or SEQ ID NO:6, an oligonucleotide which comprises a sequence capable of inhibiting translation of a mRNA into a protein, an oligonucleotide which comprises phosphorothioate linkages, an oligonucleotide which comprises between 10 and 40 consecutive nucleotides and an oligonucleotide that comprises more than 40 consecutive nucleotides. This invention provides the instant peptide, wherein the peptide is membrane permeable.

This invention provides a method of delivering an oligonucleotide into a cell comprising contacting the cell with the instant composition, under conditions permitting the composition to enter the cell and thereby deliver the oligonucleotide into the cell.

This invention provides a method of inhibiting expression of a protein in a cell comprising delivering an oligonucleotide into the cell using the method, under conditions permitting the oligonucleotide, once inside the cell, to hybridize with a nucleic acid encoding the protein

and thereby inhibit expression of the protein from the nucleic acid in the cell, for example inhibiting expression of Protein Kinase C alpha in for example a mammalian cell, which may be of human origin and/or cancerous. The nucleic acid may be a deoxyribonucleic acid or a ribonucleic acid such as a messenger ribonucleic acid.

This invention provides a method of increasing the sensitivity of a cancer cell to an anti-cancer agent e.g. paclitaxel comprising inhibiting expression of a protein in the cancer cell, for example inhibiting expression of protein kinase C alpha in for example a bladder cancer cell, using the instant method.

This invention provides a pharmaceutical composition comprising a therapeutically effective amount of either of the instant compositions and a pharmaceutically acceptable carrier.

This invention provides a method of making either of the instant compositions, comprising contacting an oligonucleotide with either of the instant peptides under conditions permitting the peptide to complex with the oligonucleotide.

This invention provides either of the instant compositions, wherein the oligonucleotide is between 10 and 40 consecutive nucleotides.

This invention also provides either of the instant compositions, wherein the oligonucleotide is longer than 40 consecutive nucleotides.

This invention provides a method of delivering an oligonucleotide into a cell comprising contacting the cell with either of the instant compositions, under conditions permitting the composition to enter the cell and thereby
5 deliver the oligonucleotide into the cell.

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2209
2210
2211
2212
2213
2214
2215
2216
2217
2218
2219
2220
2221
2222
2223
2224
2225
2226
2227
2228
2229
2230
2231
2232
2233
2234
2235
22

Brief Description of the Figures

5 Figure 1: Western Blot showing inhibition of PKC- α protein expression in T24 cells by ISIS 3521 (0.25, 0.5 and 1 μ M) (SEQ ID NO:5) complexed with l-protamine-NLS (SEQ ID NO:1). Note that chloroquine is not necessary for efficacy. The peptide itself has no effect (not shown).

10 Figure 2: This figure shows a lack of inhibition of PKC- α protein expression in T24 cells by the control oligonucleotide ISIS 4559 (2 μ M) (SEQ ID NO:7) complexed with l-protamine-NLS (SEQ ID NO:1). Note that even in the presence of chloroquine no inhibition occurs.

15 Figure 3: This figure shows increasing peptide:oligonucleotide ratio diminishes antisense efficacy in T24 cells. The optimum peptide concentration is 25 μ M.

20 Figure 4: This figure shows that the correct NLS sequence is required for efficacy of the s-protamine-NLS (SEQ ID NO:2)/oligonucleotide complex in T24 cells. When complexed with the scrambled NLS sequence (SEQ ID NO:3) no antisense activity by ISIS 3521 (SEQ ID NO:5) is detected.

25 Figure 5: This figure is a Western Blot showing inhibition of PKC- α protein expression in PC3 cells by ISIS 3521 (0.25, 1, 1.5, 2 μ M) (SEQ ID NO:5) complexed in a fixed ratio with l-protamine-NLS (SEQ ID NO:1). Note that chloroquine is necessary for efficacy. The peptide itself has no effect
30 (not shown).

Figure 6: This figure shows a lack of inhibition of PKC- α protein expression in PC3 cells by the control

oligonucleotide ISIS 4559 (2 μ M) (SEQ ID NO:7) complexed with l-protamine-NLS (SEQ ID NO:1). Note that even in the presence of chloroquine no inhibition occurs.

5 Figure 7a-b: (a) This Western blot of PC3 cells treated with l-protamine-NLS (SEQ ID NO:1) complexed with ISIS 3521 (SEQ ID NO:5) shows that reliable inhibition of PKC- α protein expression can occur with 250nM oligonucleotide concentration in the presence of 25 μ M chloroquine. (b) This
10 Western blot of PC3 cells treated with l-protamine-NLS (SEQ ID NO:1) complexed with G3139 (SEQ ID NO:6) shows that the long peptide also delivers functional G3139 antisense to cells resulting in inhibition of bcl-2 protein expression in the presence of 25 μ M chloroquine.

15 Figure 8: The antisense efficacy of l-protamine-NLS is not so heavily dependent on the integrity of NLS in PC3 cells as it is in T24 cells as shown by slight inhibition of protein expression by l-protamine-(scrambled)NLS (SEQ ID NO:4) in this figure. However, at the same peptide
20 concentration of 25 μ M the l-protamine-NLS (SEQ ID NO:1) delivery of ISIS 3521 results in a much greater inhibition of protein expression.

25 Figure 9: The antisense efficacy of s-protamine-NLS (SEQ ID NO:2) is dependent on the integrity of NLS in PC3 cells as shown by the lack of inhibition of protein expression by s-protamine-(scrambled)NLS (SEQ ID NO:3) in this figure. At the same peptide concentrations of 10 μ M and 6.7 μ M the s-
30 protamine-NLS (SEQ ID NO:2) delivery of ISIS 3521 (SEQ ID NO:5) results in a much greater inhibition of protein expression.

Detailed Description of the Invention

The following definitions are presented as an aid in understanding this invention:

5

A	-	Adenine;
C	-	Cytosine;
DNA	-	Deoxyribonucleic acid;
EGTA	-	Ethylene glycol-bis(beta-aminoethyl
10		ether)-N,N,N',N'-tetraacetic acid;
ER	-	Endoplasmic reticulum;
FBS	-	Fetal bovine serum;
G	-	Guanine;
HIV	-	Human immunodeficiency virus;
15		HPLC - High pressure liquid chromatography;
ICAM	-	Intercellular cell adhesion molecule;
l-protamine	-	long protamine peptide;
MEM	-	Modified Eagle's medium;
mRNA	-	Messenger ribonucleic acid;
20		NLS - Nuclear localization signal;
PBS	-	Phosphate buffered saline;
PKC- α	-	Protein kinase C alpha;
RPMI media	-	Rockwell Park Memorial Institute media;
s-protamine	-	Short protamine peptide;
25		SV40 - Simian virus 40;
T	-	Thymine;
TBS	-	Tris buffered saline; and
Trp	-	Tryptophane.

30 "Antisense oligonucleotide", as used herein, refers to oligonucleotides which are complementary to a specific DNA or RNA sequence which, once introduced into a cell, the combine with natural sequences produced by the cell to form

duplexes. These duplexes can then block either further transcription or translation.

5 "Complex", as used herein refers to an association of a peptide with an oligonucleotide.

10 "Phosphorothioate", when applied to an oligonucleotide, shall mean an oligonucleotide in which a sulfur atom replaces one or more of the non-bridging oxygen atoms in one or more phosphodiester linkages, i.e. an oligonucleotide having one or more phosphorothiodiester linkages.

15 This invention provides a peptide comprising consecutive amino acids, the sequence of which amino acids is shown in SEQ ID NO: 2. For example, the peptide per se, a peptide with 1-20 amino acids at either or both ends, a peptide with more than 20 amino acids at either or both ends. Preferably the peptide is membrane permeable.

20 This invention also provides a composition comprising a complex between the peptide and an oligonucleotide. Desirably the oligonucleotide comprises from about 10 to about 40 consecutive nucleotides and has a sequence capable of inhibiting translation of a mRNA into a protein.
25 Preferably the oligonucleotide comprises one or more phosphorothioate linkages. In one embodiment the oligonucleotide has more than 40 consecutive nucleotides. This invention also provides the instant composition further comprising an aqueous carrier, e.g. salines including
30 phosphate buffered saline.

This invention further provides a method of delivering an oligonucleotide into a cell comprising:

a) first contacting the cell with a lysosomotropic agent, and

b) then contacting the cell with the composition, under conditions permitting the composition to enter the cell and thereby deliver the oligonucleotide into the cell. In one embodiment the lysosomotropic agent is chloroquine. In alternative embodiments the lysosomotropic agent is any lysosomotropic amine such as dendrimers, poly-l-lysine, porphyrins. In one embodiment the oligonucleotide comprises consecutive nucleotides having the sequence shown in SEQ ID NO:5. In another embodiment the oligonucleotide comprises consecutive nucleotides having the sequence shown in SEQ ID NO:6. Desirably the oligonucleotide is an antisense oligonucleotide.

This invention still further provides a method of inhibiting expression of a protein in a cell comprising delivering an oligonucleotide into the cell, under conditions permitting the oligonucleotide, once inside the cell, to hybridize with a nucleic acid encoding the protein and thereby inhibit expression of the protein from the nucleic acid in the cell. In one embodiment the protein is Protein Kinase C alpha. Desirably the cell is of mammalian origin, preferably of human origin. In one embodiment the cell is a cancer cell e.g. a bladder cancer cell, or any human cancer cell. The nucleic acid may be a deoxyribonucleic acid, or a ribonucleic acid, particularly a messenger ribonucleic acid.

In addition this invention provides a peptide comprising consecutive amino acids, the sequence of which amino acids is shown in SEQ ID NO: 1. Preferably the peptide is membrane permeable.

This invention also provides a composition comprising a complex between the peptide and an oligonucleotide. Desirably the oligonucleotide comprises from about 10 to about 40 consecutive nucleotides and has a sequence capable of inhibiting translation of a mRNA into a protein. Preferably the oligonucleotide comprises one or more phosphorothioate linkages. In one embodiment the oligonucleotide has more than 40 consecutive nucleotides. This invention also provides the instant composition further comprising an aqueous carrier, e.g. salines including phosphate buffered saline.

This invention further provides a method of delivering an oligonucleotide into a cell comprising contacting the cell with the second composition, under conditions permitting the composition to enter the cell and thereby deliver the oligonucleotide present in the composition into the cell. In one embodiment the oligonucleotide comprises consecutive nucleotides having the sequence shown in SEQ ID NO:5. In another embodiment the oligonucleotide comprises consecutive nucleotides having the sequence shown in SEQ ID NO:6. In one embodiment the conditions comprise contacting the cell with a lysosomotropic agent prior to contacting the cell with the composition. In one embodiment the lysosomotropic agent is chloroquine. In alternative embodiments the lysosomotropic agent is any lysosomotropic amine such as dendrimers, poly-l-lysine, porphyrins.

This invention still further provides a method of inhibiting expression of a protein in a cell comprising delivering an oligonucleotide into the cell, under conditions permitting the oligonucleotide, once inside the cell, to hybridize with a nucleic acid encoding the protein and thereby inhibit

expression of the protein from the nucleic acid in the cell.
In one embodiment the protein is Protein Kinase C alpha.
Desirably the cell is of mammalian origin preferably of
human origin. In one embodiment the cell is a cancer cell
5 e.g. a bladder cancer cell or any human cancer cell. The
nucleic acid may be a deoxyribonucleic acid or a ribonucleic
acid, preferably a messenger ribonucleic acid.

This invention also provides a method of increasing the
10 sensitivity of a cancer cell to an anti-cancer agent by
inhibiting expression of a protein in the cancer cell using
the instant method. In one embodiment the anti-cancer agent
is paclitaxel. In one embodiment the cancer cell is a
bladder cancer cell. In one embodiment the protein is kinase
15 C alpha. In other embodiments the cancer cell is any human
cancer cell.

This invention also provides a pharmaceutical composition
comprising a therapeutically effective amount of either of
20 the instant compositions and a pharmaceutically acceptable
carrier. Various of such carriers known to those skilled in
the art. For example, the carrier may be a membrane-
permeable cationic reagent, a polyamidodendrimers;
transferrin polylysine; polyglycolic acid co-polymers and
25 any delivery in polymers that can be used to
nanoencapsulate, such as polylactic acid, aqueous and
nonaqueous gels, creams, multiple emulsions, microemulsions,
liposomes, ointments, aqueous and nonaqueous solutions,
lotions, aerosols, hydrocarbon bases and powders, and can
30 contain excipients such as solubilizers, permeation
enhancers (e.g., fatty acids, fatty acid esters, fatty
alcohols and amino acids), and hydrophilic polymers (e.g.,
polycarbophil and polyvinylpyrrolidone, transdermal

enhancers, transmucosal delivery systems such as patches, tablets, suppositories, pessaries, gels and creams, which can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid). Injectable systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprolactones and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprolactone.

Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

This invention provides a method of making either of the instant compositions, comprising contacting an oligonucleotide with either of the instant peptides under conditions permitting the peptide to complex with the oligonucleotide.

This invention provides either of the instant compositions, wherein the oligonucleotide is longer than 40 consecutive nucleotides.

This invention provides a method of delivering an oligonucleotide into a cell comprising contacting the cell with either of the instant compositions, under conditions permitting the composition to enter the cell and thereby
5 deliver the oligonucleotide into the cell. Such delivery may comprise gene delivery.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the
10 art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

4350000

Experimental Details

The antisense oligonucleotide we have employed is the 20-mer phosphorothioate antisense PKC- α , first developed by Dean and colleagues (13), named ISIS 3521 (SEQ ID NO:5). This, and the 18-mer antisense bcl-2 oligonucleotide G3139 (SEQ ID NO:6) that we have also employed in this study, are widely considered to be sequence-specific inhibitors of gene expression. Because of this property, they are both well suited for the evaluation of a novel delivery strategy.

Peptide synthesis

We have designed peptides in a attempt to satisfy the major requirements for efficient gene delivery and antisense activity. These requirements include: the ability to cross the cell membrane, the high affinity and specificity for oligonucleotides, and nuclear addressing. The peptides also should have a Trp residue which can act as an intrinsic probe for monitoring the interactions with oligonucleotides. The peptides were designed as bifunctional peptides.

This invention discloses peptides composed of a hydrophobic N-terminal DNA-binding domain which is a protamine fragment required for efficient crossing of the cell membrane, and a hydrophilic C-terminal nuclear localization signal (NLS) derived from the SV40 large T-antigen, required for the nuclear targeting.

There is a large family of nuclear localization signals, but we chose the simian virus SV40 large T-antigen nuclear localization sequence because it has been shown to enhance

cellular uptake of DNA (8,9). The peptide employed here consists of a basic stretch of amino acids containing five consecutive positively charged residues which are necessary for nuclear import. Successful transfection of DNA into cells in culture, by using mixtures of DNA and NLS protein, has resulted in efficient DNA transfer into the nucleus and enhanced transgene expression (8,10).

The two peptides disclosed here differ in that one was designed to possess a long Arg-rich peptide with NLS; the other peptide has a shorter protamine fragment. These two peptides are respectively referred to as l-protamine-NLS (SEQ ID NO:1) and s-protamine-NLS (SEQ ID NO:2).

Peptides form complexes with oligodeoxynucleotides

The interaction of ISIS 3521 (SEQ ID NO:5), a 20mer phosphorothioate antisense oligonucleotide, with the short and long protamine-NLS peptides (s-protamine-NLS and l-protamine-NLS) was evaluated by fluorescence spectroscopy. The peptide contains a single Trp residue (position 8 of the short peptide; SEQ ID NO:2) which can be excited at 290 nm and emits fluorescent light at 330 nm. The ability of a bound oligonucleotide to quench this fluorescent emission constitutes a relatively sensitive probe for the monitoring of molecular interactions.

The titration of a 5 μ M solution of s-protamine-NLS (SEQ ID NO:2) with increasing concentrations of ISIS 3521 (SEQ ID NO:5), both in PBS and in Opti-MEM (without serum, to mimic transfection conditions), led to dramatic fluorescence quenching. At least two oligonucleotide binding modes can be easily discerned: At low oligonucleotide concentration

(relative to s-protamine-NLS peptide) a plot of the decrease in fluorescence (due to quenching) vs. oligonucleotide concentration fits an equation of the Michaelis-Menton type. A Lineweaver-Burke (double reciprocal) plot gives $K_d = 80$ nM ($R^2 = .99$). However, as oligonucleotide concentration is increased, Trp fluorescence continues to decline, but a similar plot no longer fits a Michaelis-Menton-type equation, and the binding becomes increasingly complex. Trp fluorescence is finally completely quenched (i.e., 100%) at a 4:1 molar ratio of peptide/oligonucleotide.

Protamine-NLS peptides deliver oligonucleotides to the cell nucleus

The ability of the protamine-NLS peptides to deliver the oligonucleotides into the cell nucleus was evaluated by fluorescence confocal microscopy. 5'-fluorescein-labeled ISIS 3521 (2 μ M) (SEQ ID NO:5) was complexed with s-protamine-NLS (SEQ ID NO:2) at a peptide/oligonucleotide charge ratio 3.15:1 and 2.37:1 (2 μ M oligonucleotide; 8 μ M and 6 μ M peptide concentrations, respectively) and incubated with T24 cells pretreated for 15 min with chloroquine (25 μ M in Opti-MEM). The peptide effectively delivered fluoresceinated oligonucleotide to the cell nucleus after an incubation time of 24 hr. In the absence of peptide, oligonucleotides were internalized poorly by the cells, with most of the internalized material accumulating in a punctate pattern, signifying localization in vesicular structures (endosomes/lysosomes).

Under the same conditions and reagent concentrations, the control peptide, s-protamine-scrambled NLS (SEQ ID NO:3), also promotes the delivery of the oligonucleotide to the

nucleus. Significantly, however, when delivered in this fashion, the oligonucleotide does not downregulate the expression of PKC- α protein as determined by Western blotting.

5

Peptide-oligodeoxynucleotide complexes demonstrate antisense activity

Short peptide

10 Experiments using the protamine-NLS peptides to deliver antisense oligonucleotides were performed in T24 bladder carcinoma and PC-3 prostate carcinoma cells. In addition to the 20-mer ISIS 3521, the 18-mer phosphorothioate oligonucleotide G3139 (SEQ ID NO:6) was also employed. G3139 is targeted to the first six codons of the human bcl-2 open reading frame. The optimum incubation time of the cells with the peptide/oligonucleotide complexes was 24 hr. For experiments with s-protamine-NLS (SEQ ID NO:2) cells were pretreated with 25 μ M of chloroquine for 15 min. The expression of PKC- α protein was assayed by Western blotting after 24 hr, while the expression of bcl-2 protein in PC3 cells was assayed similarly after a further 48 hr incubation in RPMI media containing 10% FBS. In both cases, the incubations were performed in the continuous presence of the peptide/oligonucleotide complexes.

The s-protamine-NLS (SEQ ID NO:2), when complexed to ISIS 3521 (2 μ M) (SEQ ID NO:5) at a 4:1 or 3:1 molar ratio (3.15 and 2.37 charge ratio, respectively) of peptide/oligonucleotide yielded significant antisense activity in both T24 (Fig 4.) and PC-3 cells (Fig. 9). By Western blotting and scanning densitometry, the expression of the PKC- α (T24 and PC-3 cells) and Bcl-2 (PC3 cells)

proteins was reduced by approximately 80%, 80% and 70%, respectively. Naked oligomer (i.e., not complexed with peptide) at the identical concentration (2 μ M) + 25 μ M chloroquine preincubation was completely ineffective, most likely because of the sequestration of the oligonucleotides in endosomes/lysosomes, as demonstrated by confocal microscopy. A control, scrambled phosphorothioate oligonucleotide (ISIS 4559, SEQ ID NO:7) complexed with the s-protamine-NLS (SEQ ID NO:2) and a complex of ISIS 3521 (SEQ ID NO:5) with s-protamine-scrambled-NLS (SEQ ID NO:3) under identical conditions (+ 25 μ M chloroquine preincubation) did not produce any reduction in target PKC- α protein levels in PC3 cells (Fig.9). Furthermore, 25 μ M chloroquine alone did not downregulate PKC- α expression in either cell line.

In addition, G3139 (SEQ ID NO:6) when complexed to s-protamine-NLS (SEQ ID NO:2) was "antisense" active in PC-3 cells. Similar controls as employed above (scrambled oligonucleotide (see fig. 6), scrambled NLS sequence, chloroquine alone) were all totally inactive. Approximately 70% inhibition of Bcl-2 expression could be obtained with 4:1 and 3:1 molar ratios of peptide/oligonucleotide.

Long peptide

The ability of 1-protamine-NLS (SEQ ID NO:1) to diminish PKC- α and Bcl-2 protein expression in T24 and PC-3 cells was also evaluated. Complexes of this peptide with ISIS 3521 (see Fig. 1) and G3139 oligonucleotides (see Fig. 7b) were active over approximately the same concentration range in both cell lines. In T24 cells, expression of PKC- α (Fig. 1) and Bcl-2 proteins were reduced. However it is notable that chloroquine pretreatment was not required for inhibition of

protein expression in T24 cells when the 1-protamine-NLS was used to deliver the oligonucleotide (Fig. 1). Controls of 1-protamine-NLS complexed with ISIS 4559 did not inhibit protein expression (see Fig. 2). The ratio of peptide:oligonucleotide affected the antisense efficacy (see Fig. 3).

In PC-3 cells protein expression was reduced by the peptide-oligonucleotide complex (see Fig. 5 and Fig. 7a), but the cells did require pre-treatment with 25 μ M chloroquine for this effect to be observed. Controls, such as ISIS 3521 complexed with 1-protamine-scrambled NLS (SEQ ID NO:4) had a reduced effect (see Fig. 8) and ISIS 3521 (SEQ ID NO:5) alone did not have antisense effects.

It is clear that other agents similar to chloroquine could be used in place of chloroquine to effect the same purpose. Also oligonucleotides longer than 40 nucleotides could be complexed with peptides for cell delivery.

Downregulation of PKC- α increases sensitivity of T24 bladder carcinoma cells to paclitaxel

As a measure of the effectiveness of the peptide-delivered antisense inhibition of PKC- α the cells treated with peptide were exposed to paclitaxel, a compound commonly used in cancer therapy to inhibit microtubule depolymerization.

After 3 days of paclitaxel treatment T24 cells were treated for 24 hours with s-protamine-NLS (8 μ M)/ISIS 3521 (2 μ M) complexes (3.15:1 charge ratio) after pretreatment with 25 μ M chloroquine. ISIS 3521, s-protamine-NLS peptide (SEQ ID NO:2) and 25 μ M chloroquine alone had no effect on cell

viability. Complexes of s-protamine-scrambled-NLS/ISIS 3521 and s-protamine-NLS/scrambled ISIS 3521 also did not reduce cellular viability. Only treatment of the cells with s-protamine-NLS/ISIS 3521 was able to reduce cellular viability as measured by the reduction in cell proliferation determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Materials and Methods

Peptide Synthesis: Peptide synthesis was carried out on a Rainin PS3 Automated Solid Phase Peptide Synthesiser using standard Fmoc chemistry. Standard deprotection and cleavage methods were employed, by treating the peptide with a mixture containing 95% TFA, 2.5% triisopropylsilane and 2.5% H₂O (4 ml), for 2 hours at room temperature with constant stirring. The peptide was then isolated by ether extraction.

Purification and Characterization of the Peptides: Reverse phase HPLC was carried out using a Vydac 218TP C18 protein and peptide RP 5M column (4.6 x 250mm) on a Waters 996 Photodiode Array Detector. Samples were monitored at 254 nm and a mobile gradient buffer system used. Mobile phase A was 0.1M trimethylammonium bicarbonate (pH 7.0) and mobile phase B was HPLC grade acetonitrile.

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) was carried out on a Bruker Biflex spectrometer and all data was collected and analysed using Solaris software. Peptides were analyzed in positive linear mode as 50 pM in 3:7 acetonitrile/water and 0.1% trifluoroacetic acid. Molecular weight of peptide SEQ ID NO:2 is 2998.97 (M/Z⁺¹ 3000). Molecular weight of peptide SEQ ID NO:1 is 4580.0 (M/Z⁺ 4580.9).

Cell culture: T24 cells were grown in McCoy's 5A medium (Gibco BRL, Grand Island, NY), containing 10% (v/v) heat inactivated (56°C) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), supplemented with 25 mM Hepes, 100 units/mL penicillin G sodium and 100 g/mL streptomycin sulfate. PC-3

cells were grown in RPMI 1640 medium (Gibco BRL), containing 10% (v/v) FBS, to which was added 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non essential amino acids. Stock cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

Reagents: The anti-PKC-alpha mouse monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). The anti-bcl-2 mouse monoclonal antibody was purchased from Dako (Carpinteria, CA). The anti-mouse horseradish peroxidase conjugated secondary antibody was from Amersham (Arlington Heights, IL).

Synthesis of oligonucleotides: Phosphorothioate oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) model 380B DNA. Following cleavage from controlled pore glass support, oligodeoxynucleotides were base deblocked in ammonium hydroxide at 60°C for 8 hours and purified by reversed phase HPLC [0.1% TFA, TEAB/acetonitrile. PRP-1 support]. Oligomers were detritylated in 3% acetic acid and precipitated with 2% lithium perchlorate/acetone, dissolved in sterile water and reprecipitated as the sodium salt from 1 M NaCl/ethanol. Concentrations were determined by UV spectroscopy.

The sequences of the oligonucleotides used were: ISIS 3521 (targeted to the 3' region of the PKC- α mRNA), 5'-GTTCTCGCTGGTGAGTTTCA-3' (SEQ ID NO:5); G3139 (targeted to the first 6 codons of the human bcl-2 open reading frame), 5'-TCTCCCAGCGTGCGCCAT-3' (SEQ ID NO:6). The sequence of the control scrambled oligonucleotide (ISIS 4559) was 5'-GGTTTTACCATCGGTTCTGG-3' (SEQ ID NO:7).

Nature of the complex: The nature of the complex is not yet fully elucidated, it is thought that hydrogen of the positively charged guanidinium functional groups within it result in electrostatic interactions with the negatively charged phosphate backbone of the nucleic acid molecule.

Treatment of cells with oligonucleotide-peptide complexes: Cells were grown in six-well plates until 65-75% confluent. The peptides, at the stated peptide/oligonucleotide molar ratios, were diluted in 500 μ L of Opti-MEM medium and oligonucleotide was then added up to the required concentration of 2 μ M. The solution was mixed gently and incubated at room temperature for 30 min to allow oligonucleotide-peptide complexes to form. Then the complexes were overlaid onto the cells, which had been rinsed with Opti-MEM medium and pretreated with 500 μ L chloroquine (final concentration, 25 μ M in Opti-MEM) for 15 min. The cells were then incubated at 37°C for 24 hr (for PKC- α) or re-fed with complete McCoy's 5A media containing 10% FBS and allowed to incubate for an additional 72 hr (for bcl-2) before cell lysis and extract preparation.

Fluorescence titrations: Fluorescence experiments were performed on a luminescence spectrometer, Aminco Bowman series 2, SLM Aminco (Urbana, IL). The intrinsic tryptophan fluorescence of peptide was excited at 290 nm, and the emission spectrum was recorded between 310 and 410 nm with a spectral bandpass of 4 nm. A fixed concentration of protein (5 μ M) was titrated by increasing the concentration of oligonucleotide (in a range of 0-1 μ M) at room temperature in PBS buffer. Curve fitting was performed with the Grafit program (Excel Software).

Confocal microscopy: Cells were seeded in glass bottom microwells (MatTec Corp., MA), and treated with peptide-oligodeoxynucleotide complexes at 37°C for 24 hr. Cellular internalization was examined using an LSM 410 laser scanning
5 confocal microscope (Zeiss, Thornwood, NY) equipped with a krypton/argon laser and attached to a Zeiss Axiovert 100 TV microscope. The 515-540 nm bandpass for fluorescein was used. Z-series were taken of a 1 to 2 micron optical section at 2 μ m intervals. For measurements, a maximum projection of
10 all sections was employed.

Western Blotting: Cells were treated with peptide-oligodeoxynucleotide complexes, scraped, washed with cold PBS and then extracted in 40-50 mL of lysis buffer [50 mM
15 Tris-HCl, pH 7.5; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 50 μ g/mL Pefablock™ SC; 15 μ g/mL aprotinin, leupeptin, chymostatin, and pepstatin A; 1 mM Na₃VO₄; 1 mM NaF] at 4°C for 1 hr. Cell debris was removed by centrifugation at 14,000X g for 20 min at 4°C. Protein
20 concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA).

Aliquots of cell extracts containing 20-30 μ g of protein were resolved by 10% or 12% SDS-polyacrylamide gel
25 electrophoresis and transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL). Filters were incubated at room temperature for 1-2 h in Blotto A [5% non-fat milk powder in TBS-T: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (for PKC- α) and 5% BSA in PBS, 0.5% Tween (for Bcl-
30 2)] and then probed overnight at 4°C with 1:1000 dilution of anti-PKC- or 1:500 dilution of anti-Bcl-2 in Blotto A. After washing in TBS-T or PBS-T buffer (3 X 7 min, room

temperature), filters were incubated for 1 h at room temperature in 5% milk/TBS-T or 5% milk/PBS-T buffer containing a 1:3,000 dilution of peroxidase-conjugated anti-mouse secondary antibody. The filters were then washed (3 X 10 min, room temperature), and ECL was performed according to the manufacturer's instructions (Amersham).

Cellular viability assay: Cells were seeded in 96-well tissue culture plates and treated the next day with oligonucleotide-peptide complexes for 24 hr. After 3 days treatment with paclitaxel (at the indicated concentrations as described in the Results), cellular viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated for 4 hr at 37°C, 5% CO₂ with 0.5 mg/mL MTT in complete media. An equal volume of solubilization solution (10% SDS in 0.01 M HCl) was then added and allowed to incubate overnight at 37°C. After the formazan crystals were dissolved, the plates were read on a Dynatech MR600 Microplate Reader at 540 nm.

Statistical analysis of the results was performed using the Analysis ToolPack provided by Microsoft Excel. A Student's two-sample t-test, assuming unequal variances, was used to determine the equality of the means of two samples. The confidence level was 0.05.

References

1. Arar, K., Monsigny, M., and Mayer, R. (1993) Tetrahedron Lett. 34:3087-90
- 5 2. de la Torre, B., Albericio, F., Saison-Behmoaras, E., Bachi, A., and Eritja, R. (1999) Bioconjugate Chem., 10:1005-1-12
- 10 3. Eritja, r., Pons, A., Escarceller, M., Giralt, E., and Albericio, R. (1991) Tetrahedron 47:4113-4120
4. Reed, M., Fraga, D., Schwartz, D., choller, J., and Hinrichsen, R. (1995) Bioconjugate Chem. 6, 101-108
- 15 5. Neves, C., Byk, G., Scherman, D., and Wils, P. (1999) FEBS Lett. 453:41-45
6. Lebedeva, I., and Stein, C.A. "Antisense in Cancer: Recent Advances", BioDrugs, 2000, (13), 195-216.
- 20 7. Morris, M.C., Vidal, P. Chaloin, L., Heitz, F., and Divita, G. (1997) Nucl. Acids Res. 25:2730-2736
- 25 8. Collas P, Husebye H, Alestrom P. (1996) The nuclear localization sequence of the SV40 T antigen promotes transgene uptake and expression in zebrafish embryo

nuclei. Transgenic Res. 5(6):451-8.

5 9. Collas P. and Alestrom P. (1997) Rapid targeting of plasmid DNA to zebrafish embryo nuclei by the nuclear localization signal of SV40 T antigen. Mol Mar Biol Biotechnol. 6(1):48-58.

10 10. Wienhues U, Hosokawa K, Hoveler A, Siegmann B, Doerfler W. (1987), A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells. DNA. 6(1):81-9.

15 11. Pichon C, Arar K, Stewart AJ, Dodon MD, Gazzolo L, Courtoy PJ, Mayer R, Monsigny M, Roche AC. (1997) Intracellular routing and inhibitory activity of oligonucleopeptides containing a KDEL motif. Mol Pharmacol. 51(3):431-8.

20 12. Pichon C, Roufai MB, Monsigny M, Midoux P. (2000) Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. Nucleic Acids Res. 28(2):504-12

25 13. Dean N, McKay R, Miraglia L, Howard R, Cooper S, Giddings J, Nicklin P, Meister L, Ziel R, Geiger T, Muller M, Fabbro D. (1996) Inhibition of growth of human tumor cell lines in nude mice by an antisense of oligonucleotide inhibitor of protein kinase C-alpha expression. Cancer Res. 56(15):3499-507